RESEARCH ARTICLE

Androgen responses to reproductive competition of males pursuing either fixed or plastic alternative reproductive tactics

Corinna von Kuerthy[1,*], Albert F. H. Ros[2] and Michael Taborsky[1]

ABSTRACT

Alternative reproductive tactics (ARTs), which can be plastic or fixed for life, may be characterized by distinct hormonal profiles. The relative plasticity hypothesis predicts flexible androgen regulation for adult males pursuing plastic tactics, but a less flexible regulation for males using a fixed tactic throughout life. Furthermore, androgen profiles may respond to changes in the social environment, as predicted by the social reciprocity models of hormone/behaviour interactions. The cichlid fish *Lamprologus callipterus* provides a rare opportunity to study the roles of androgens for male ARTs within a single species, because fixed and plastic ARTs coexist. We experimentally exposed males to competitors pursuing either the same or different tactics to test predictions of the relative plasticity and the social reciprocity models. Androgen profiles of different male types partly comply with predictions derived from the relative plasticity hypothesis: males of the plastic bourgeois/sneaker male trajectory showed different 11-ketotestosterone (11-KT) levels when pursuing either bourgeois or parasitic sneaker male behaviours. Surprisingly, males pursuing the fixed dwarf male tactic showed the highest free and conjugated 11-KT and testosterone (T) levels. Our experimental social challenges significantly affected the free 11-KT levels of bourgeois males, but the androgen responses did not differ between challenges involving different types of competitors. Furthermore, the free T-responses of the bourgeois males correlated with their aggressive behaviour exhibited against competitors. Our results provide new insights into the endocrine responsiveness of fixed and plastic ARTs, confirming and refuting some predictions of both the relative plasticity and the social reciprocity models.

KEY WORDS: Alternative reproductive tactics, Androgens, 11-ketotestosterone, Testosterone, Relative plasticity hypothesis, Social reciprocity models, Social environment

INTRODUCTION

Androgens are priming regulators of many behavioural, physiological and morphological features of reproduction (Nelson, 2005). They trigger sexual differentiation (Moore et al., 1998), the development of primary and secondary sexual characters (Fishelson, 1963), mating behaviour (Kindler et al., 1991), and the control of reproductive investment (e.g. spermatogenesis, Schreibman et al., 1986; Miura et al., 1992). Moreover, androgens are important regulators and modulators of alternative reproductive

tactics (ARTs) governing the transitions between different life-history stages (Oliveira et al., 2001a; Schradin and Yuen, 2011). ARTs are generally characterized by differences in behavioural, physiological and sometimes morphological traits among same-sex conspecifics, which result from disruptive sexual selection (Taborsky et al., 2008; Taborsky and Brockmann, 2010).

Large 'bourgeois' males compete by investing heavily in reproductive behaviour (Gross, 1982, 1996; Taborsky and Brockmann, 2010), growth (Wirtz-Ocaňa et al., 2013), conspicuous body ornaments (Neat et al., 2003; Candolin and Wong, 2008), extended phenotypes (Schaedelin and Taborsky, 2006, 2009), weaponry (Tschernavin, 1938), acoustic signals (Brantley and Bass, 1994) or the release of sex pheromones (Laumen et al., 1974). They commonly monopolize reproductive resources, which creates opportunities for non-courting males to exploit their effort (Taborsky, 1994, 1998, 2001; Neff et al., 2003). These 'parasitic' males may benefit from an inconspicuous appearance when approaching a nest in order to steal fertilizations from bourgeois males (Warner, 1984; Taborsky, 1994). ARTs may be fixed for life, in which case individual males permanently differentiate into pursuing one of the tactics, or they may be plastic, when individual males may reversibly or irreversibly switch tactics during their lifetime (Taborsky et al., 2008). These different patterns have been shown to be associated with diverging hormone responses in several species with ARTs (Oliveira et al., 2001b; Ros et al., 2004; Knapp and Neff, 2007; Gonçalves et al., 2008; Ros and Oliveira, 2009; Saraiva et al., 2013).

The relative plasticity hypothesis (Moore, 1991) attempts to explain the different regulatory mechanism underlying fixed and flexible phenotypes by proposing an organizational role of hormones in the case of fixed tactics (effective early in life) and an activational role of hormones if tactics are plastic (Arnold and Breedlove, 1985). Based on these assumptions, the relative plasticity hypothesis predicts that in species with ARTs, hormone profiles of males pursuing plastic alternative tactics should differ among each other in the adult stage. Experimental administration of sex steroids should promote differences in behaviour and physiology, for example, causing individuals to switch from the parasitic to the bourgeois male tactic (Ros et al., 2004). In contrast, hormone profiles of males pursuing fixed morphs are not predicted to differ in the adult stage, but instead should differ during early development. In this case, hormone administration should only influence tactic choice during early development (Moore, 1991).

Empirical support for the relative plasticity hypothesis, however, is ambiguous (Oliveira, 2005; Oliveira et al., 2008). One reason might be that androgen levels are not only affected by activational and organizational effects (relative plasticity hypothesis) but, moreover, they have also been shown to be modulated by an individual's social environment (Mazur, 1985; Wingfield et al., 1990; Hirschenhauser and Oliveira, 2006; Lacava et al., 2011). Bourgeois males usually defend and monopolize resources (contest

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competition) to get access to mates and thus face enhanced levels of social challenges. In contrast, parasitic males often compete among each other through scramble competition instead of direct contests (Toquenaga, 1990; Toquenaga and Fujii, 1990; John, 1993). Accordingly, hormone titres might differ between males pursuing divergent fixed tactics either because this is how alternative male reproductive behaviour is regulated (relative plasticity hypothesis), or because the current competitive situation triggers hormonal responses to instantaneously adjust the behavioural response to the social and reproductive environment (social reciprocal regulation, as proposed by the biosocial model of Mazur, 1985 and the challenge hypothesis of Wingfield et al., 1990; see Oliveira and Oliveira, 2014, for review). To disentangle the importance of these two models of potential regulatory influence of hormones on tactics (Fig. 1a,b) and the social environment on hormone levels (Fig. 1c), we investigated androgen responses of males pursuing fixed and flexible alternative reproductive tactics in different standardized competitive test situations. It should be noted that these two models are not mutually exclusive but may be viewed as being complementary and that their predictions partly overlap (Oliveira, 2005).

As a study system, we used the snail shell-brooding cichlid *Lamprologus callipterus* from Lake Tanganyika, East Africa. This species is unusual because males show both fixed alternative life-history pathways that are determined by a Mendelian genetic polymorphism ('bourgeois male' and 'dwarf male' pathways; Wirtz Ocana et al., 2014) and plastic tactic choice in the bourgeois life-history pathway (sneaker or nest holder behaviour; see below for a detailed description of male tactics).

We conducted two experiments: (i) to disentangle the association between androgens and the expression of alternative reproductive tactics; and (ii) to investigate the potential effect of social/reproductive context on androgen levels in male *L. callipterus* (cf. Fig. 1). In a first step, we tested predictions of the relative plasticity hypothesis by comparing androgen responses of individuals pursuing fixed and plastic alternative reproductive tactics in a test situation where males of all tactics and females were present. We
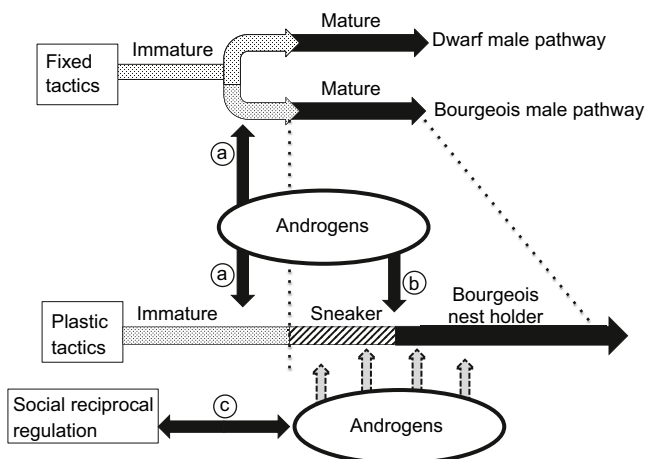
predicted that the androgen profiles of nest males and dwarf males should not differ (fixed alternative tactics) because the reproductive period of both these tactics corresponds to their final life history stage, so they should be selected to maximize investment in mating behaviour and spermatogenesis. Sneaker males, on the other hand, pursue a transient and opportunistic tactic, making the best of a bad situation: because of their medium size they can neither monopolize females (like bourgeois males) nor enter the shells for spawning (like dwarf males), which are both very successful strategies (Wirtz Ocana et al., 2014). Therefore, we predicted that the androgen levels of males pursuing the transient sneaker tactic should be lower than those of males performing the other two tactics. In a second step, we tested social reciprocity models (cf. Oliveira and Oliveira, 2014) such as the biosocial model and the challenge hypothesis, which postulate that androgen profiles are influenced by the current social and reproductive environment (e.g. Goymann, 2009; Lacava et al., 2011; Creel et al., 2013; Almeida et al., 2014). The response in androgen levels of focal males to their social/reproductive environment was tested by exposing bourgeois as well as fixed dwarf males and plastic sneaker males to a different social context, while quantifying their hormonal responses.

We measured androgen levels in both territorial and intruder males. We tested whether males of the different tactics cause a significant androgen response in territorial nest males when the former are acting as intruders. This is expected to generate a different response than intrusions of males pursuing the same tactic, because parasitic sneaker males usually pose a different and typically much smaller threat (loss of some paternity) for the bourgeois male than competitors of the same tactic (Maan and Taborsky, 2008). Not only bourgeois males, but in fact individuals of each male tactic are predicted to incur the highest level of competition from rivals pursuing the same tactic (von Kuerthy and Taborsky, 2016), which results in negative frequency dependence (Maynard Smith, 1974; Gross, 1991).

To recapitulate, since the social challenge regimes are highly asymmetric between territorial bourgeois males and subordinate bourgeois or parasitic males, we predicted that we would find androgen levels associated with aggressive and reproductive behaviour to be higher in territorial bourgeois males compared with similar-sized subordinate intruder males of the bourgeois tactic and smaller parasitic sneaker males. In contrast to this, the relative plasticity hypothesis predicts that hormone profiles are similar between bourgeois males and the fixed alternative dwarf males (Moore, 1991). When comparing the different competitive situations in order to test the social reciprocity models, we hypothesized that androgen levels of bourgeois males should increase from the non-breeding baseline level (after 5 days in isolation) to a situation when they are exposed to ovulating females. Further, as proposed by the challenge hypothesis (Wingfield et al., 1990) and confirmed in several cichlid species (Hirschenhauser et al., 2004), dominant bourgeois males holding breeding resources and females were expected to respond with a further increase in androgen levels above the breeding baseline levels when challenged by male intruders. Here, we test whether this increase is modulated by the type of male competitors they are facing.

Fig. 1. Scheme of the two genetically fixed life history pathways of *Lamprologus callipterus*. The potential regulatory influence of androgens according to the relative plasticity and social reciprocity hypotheses are indicated. One of the two pathways, the bourgeois male pathway, also includes a plastic choice possibility between sneaker and nest holder tactics (see dashed lines). The relative plasticity hypothesis predicts that androgen levels of males pursuing alternative reproductive tactics reflect organizational (a) or activational (b) effects, whereas the social reciprocity models predict that androgen levels relate to the social environment of an individual (c).

MATERIALS AND METHODS
Study system
Large bourgeois nest males of *Lamprologus callipterus* Boulenger 1906 collect empty snail shells and defend them against intruders, thereby providing protection to females breeding inside these shells (Sato, 1994; Sato et al., 2004). During reproduction, nest holders are

frequently challenged by other potential nest males trying to take over their nest and by the interference of males pursuing one of two alternative parasitic mating tactics (Taborsky, 1998, 2001; Sato et al., 2004; Schütz et al., 2012). Firstly, sneaker males of the bourgeois male pathway try to steal fertilizations from nest owners by opportunistically darting into a nest male's territory during spawning. When passing a certain threshold size (Schütz and Taborsky, 2005) and after accumulating sufficient energy stores (von Kuerthy et al., 2015), these plastic males may switch to nest male status. The second parasitic tactic is employed by the much smaller and genetically fixed dwarf males. Dwarf males attempt to steal fertilizations from bourgeois males by wriggling past a spawning female into the tip of the shell (Sato et al., 2004; Taborsky, 1998, 2008), from where they may fertilize the majority of the eggs (Wirtz Ocana et al., 2014).

Housing conditions
Our experiment was conducted at the Department of Behavioural Ecology of the University of Bern. We used wild-caught (W) individuals of *L. callipterus* from Lake Tanganyika and F1 individuals bred in our laboratory (similar and balanced W:F1 numbers). Wild-caught fish were fed, handled and kept in our aquaria for more than a year before the experiment commenced. During the experiment we did not detect any significant behavioural differences between wild-caught and F1 fish within all three types of tactics. Apparently, wild-caught fish had completely habituated to the laboratory conditions. Additionally, we used similar proportions of wild and F1 fish for our experiment, thereby avoiding any systematic bias potentially resulting from these two different origins. The water temperature in experimental tanks was kept constant at 27±2°C with a 13 h light:11 h dark cycle. Salinity and water chemistry matched the values of Lake Tanganyika closely (Taborsky, 1984) and all fish received food *ad libitum* twice a day (either TetraMin dry food or fresh *Artemia*, *Daphnia* or Mosquito larvae).

Experimental design
Focal nest males of *L. callipterus* were placed in a closed tank compartment of ∼500 litres (∼130×63×63 cm) within a 1000 litre

tank, which provided several hiding opportunities. They were introduced into the compartment 1 week prior to the start of the experiment. Six empty snail shells of *Neothauma tanganicense*, the standard breeding substrate of *L. callipterus*, were introduced into the compartment on the day before the experiment in order to induce territorial behaviour in focal nest males.

Induction of ovulation in females
On the experimental day, two ovulating females were introduced to the focal nest male's compartment at 08:45 h (±30 min, Fig. 2). Ovulation in *L. callipterus* females was induced by treating with LHRH (des-Gly10[D-Ala6]-luteinizing hormone releasing hormone ethylamide; Sigma-Aldrich, L-4513) following an established protocol (Hirschenhauser et al., 2002, 2004) using a stock solution of LHRH mixed with freshwater teleost Ringer solution. A total of 17 µg LHRH per 10 g female body mass was injected intraperitoneally and resulted in an ovulation peak after approximately 48 h (Hirschenhauser et al., 2002). No adverse effects of the treatment were observed at any time. Ovulation in females was checked by inspecting the belly and genital papilla swelling (Trewavas, 1983). After introduction of the female to the experimental tank of the nest male (Fig. 2), courtship always commenced within 15 min.

Experimental phases
The experiment comprised two phases (Fig. 2). A pre-exposure phase where the nest males were initially kept in the experimental compartment with only two females for 1 h. This phase without male competitors therefore served for initiating territorial and courtship behaviours. The second competitive or non-competitive exposure phase followed after 1 h when each focal nest male was exposed to five possible test situations. In the non-competitive test situation (exposure phase 1, Fig. 2), the focal nest male was continuously kept with only two females for the entire duration of the experiment (2 h). In the competitive male-exposure phases, the focal nest male was confronted with a size-matched potential nest male (exposure phase 2), two smaller sneaker males (exposure phase 3), two dwarf males (exposure phase 4), or one sneaker and one dwarf male (mix; exposure phase 5). The mixed test situation
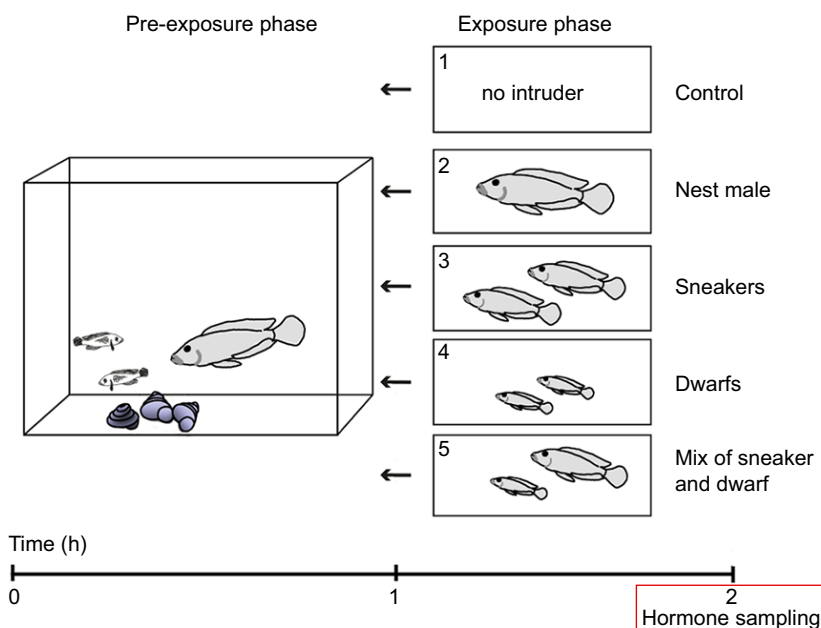

Fig. 2. Experimental design. In the pre-exposure phase (left), the male spends the first hour with two females in the experimental compartment to initiate territorial and courtship behaviours. In the exposure phase (right) at the start of the second hour, the nest male is exposed to one of five test situations (1–5). (1) No intruder male (only two females present); (2) with a potential nest male as intruder; (3) with two sneaker males; (4) with two dwarf males; or (5) a mix of one sneaker and one dwarf male. At the end of hour 2, focal males of all types were sampled for androgens.

(Fig. 2), in which individuals of all three male tactics were present at the same time, was used for a direct comparison of behavioural interactions and the androgen levels of all different male tactics.

The total length of an individual (TL in cm) refers to the length of a fish measured from the tip of the snout to the end of the caudal fin. Our focal nest males (TL: 10.0–12.6 cm) were tested consecutively in each of the five test situations (Fig. 2), with 1 week isolation intervals between the subsequent tests. All focal individuals were weighed (total body mass) to the nearest 0.1 g. Intruder males were chosen using the following criteria: intruders of the nest male type (TL: 10.0–12.1 cm) were always chosen to be size matched (±5 mm) and as similar in mass as possible. Parasitic sneaker males were significantly smaller than nest males (TL: 6.6–9.9 cm) but were sexually mature (based on previous behavioural observations). Within the sneaker and the dwarf male (TL: 3.3–4.3 cm) test situations, the two parasitic male intruders of the same tactic type were always size-matched. In the mixed tactic test situation, the focal nest males always received new parasitic intruder males that they had not previously encountered in another test situations. During the entire experiment, focal individuals were allowed to freely interact with each other. All test situations were conducted in randomized order to eliminate sequence effects. We used digital cameras to record and analyse all interactions of focal individuals. Additionally, up to 10 live scans per day were performed to check for any aberrant signs in physical appearance (e.g. wounds, pale body colour) and any suspicious behaviour (e.g. hiding, not moving), which might indicate potential indisposition of focal individuals. We never observed any indication of such an indisposition or had any other reason to intervene during the experiment.

Behavioural observations

In all five test situations (Fig. 2, exposure phases 1–5), we quantified aggressive behaviours of the nest owner directed towards intruder males (overt aggression: chasing and biting; restrained aggression: fast approaches, head down postures, fin spreading, opercula spreading) and courtship behaviours directed towards females (zigzag swimming, bumping, shell mouthing), nest maintenance behaviours (shell manipulation, sand transport) and passive behaviour (lying on ground, or floating above ground). Behaviours were analysed using The Observer 5.0 software (Noldus, Wageningen, The Netherlands). Each focal nest male was observed twice for 5 min each, 20 and 40 min after the start of the respective exposure phase (Fig. 2). In most cases, the focal individuals were distracted by the experimenter upon their introduction into the experimental compartment and only after ∼10–15 min did they start to relax, explore the compartment and interact with other individuals (courtship, but also aggressive behaviours).

Androgen survey

We measured the two major androgens: 11-ketotestosterone (11-KT) and testosterone (T). 11-KT has been shown to be the major biologically active androgen in teleosts inducing the development of male secondary sexual characters (Oliveira et al., 2001b; Ros et al., 2004) and is involved in the regulation of territorial and courtship behaviour, and the induction of spermatogenesis (Uglem et al., 2002; Weltzien et al., 2002; Ros et al., 2004; Saraiva et al., 2013). Testosterone levels are typically relatively high in species with sequential or reversible tactics (Oliveira, 2005). Because both fixed and plastic ('sequential or reversible') male tactics occur in our species, we investigated whether testosterone shows different relationships in males pursuing different male tactics. Thus, although T and 11-KT are both androgens, they offer different mechanistic pathways to tune ARTs to the social environment.

Hormone measurements from fish holding water

To allow repetitive, non-invasive measurements after different test situations and to avoid invasive blood sampling, steroids were measured from fish holding water (Hirschenhauser et al., 2002, 2004; Oliveira et al., 2002, 2003; Scott and Ellis, 2007; Scott et al., 2008). We collected fish holding water samples from focal nest males and their competitors after they had spent 1 h in the respective test situation (exposure phases 1–5, Fig. 2). Previous work with these cichlids has shown that a period of 1 h is sufficient to measure clear endocrine responses to a challenge posed by intruding males (Hirschenhauser et al., 2004). Additionally, we took control samples from non-breeding bourgeois males after 5 days in isolation. All individuals in the different treatments were exposed to the same sampling procedures. Fish were sampled in squared glass containers with rounded corners (18×12×12 cm) filled with 1 litre of blank water from an empty, clean supply tank. Individuals were sampled for 1 h, between 11:00 h and 12:00 h (±30 min), to standardize for diurnal variation of hormone levels. The glass containers were placed in a Styrofoam box so that the tested individuals were not disturbed during the sampling period. From the 1 litre of well-stirred sampling water, 600 ml were filtered through folded paper filters (Whatman 595 1/2) and eventually passed through a C18 extraction column (Merck, Sep Pak RP-18). Columns had been activated before with 2 ml ethanol and 2 ml pure H_2O. Columns were kept in the freezer at −20°C until they were further processed in the laboratory.

Hormones are released passively into the water via the gills (Ellis et al., 2004, 2005; Scott and Ellis, 2007) and indirectly by active excretion via the urine (such as sulphates in urine and glucuronides in bile). To be excreted via urine, hormones are conjugated before release. We therefore deconjugated the sample before measuring the androgens and report both the non-conjugated (free, more hydrophobic) fraction as well as the conjugated (more hydrophilic) fraction. Based on time-release studies, it has been shown that the conjugated fraction represents an integration of hormone levels during the period that urine is produced (30 min to 1 h), therefore covering a larger time window than the more immediate fluctuations of steroids in the blood represented in the free fraction (Bender et al., 2006; Scott and Ellis, 2007; Scott et al., 2008). The conjugated fraction therefore provides additional information on steroid profiles. Also, some conjugated steroids are used by fish as pheromones in order to send a signal to a potential competitor or female and thus measuring the conjugated fraction may provide hints on potential signalling patterns (Almeida et al., 2003, 2005; Barata et al., 2007; Hirschenhauser et al., 2008).

We extracted the free fraction and the corresponding sulphates and glucuronides, following established procedures to measure steroids from fish holding water and urine (for validation of this method, see Scott and Sorensen, 1994; Oliveira et al., 1996; Sorensen et al., 2005; Scott et al., 2008). The sulphates and glucuronides were extracted separately in two steps. First, we chemically extracted the sulphated fraction after overnight hydrolyses with trifluoroacetic acid in acetyl acetate (1.4:100). We then extracted the glucuronated fraction enzymatically using β-glucuronidase/aryl sulphatase (Merck). We used enzyme immunoassays (EIAs) to measure the free fraction and the conjugates (glucuronides and sulphates) of the steroids 11-ketotestosterone (KT) and testosterone (T). All samples were analysed in duplicate and were randomly distributed over the different EIA plates. Crossreactivity as reported by the supplier (Cayman Chemical) was 0.01% or less for 11-KT antiserum with testosterone. For the 11-KT EIAs, we had an average intra-assay coefficient of variability of % CV=5.3. For the EIAs of testosterone, the average intra-assay coefficient was % CV=5.61. CVs were calculated from the sample concentrations (not the raw optical
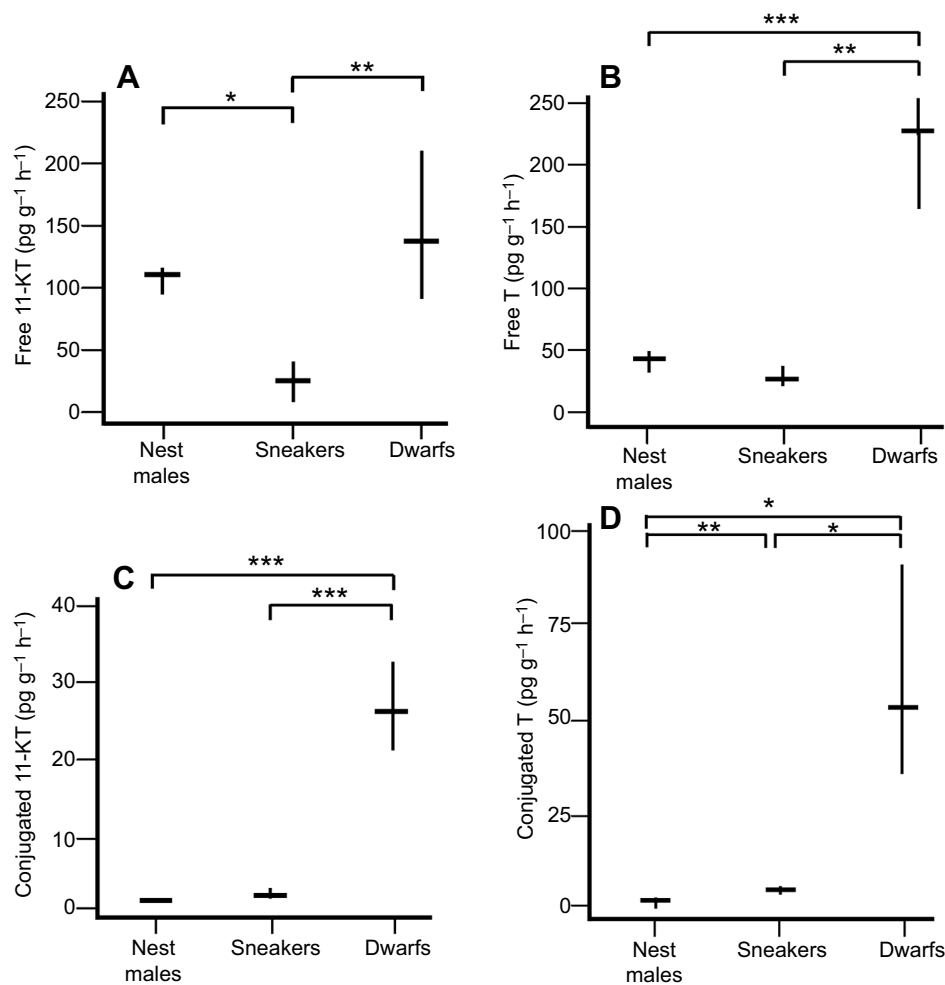
Fig. 3. Androgen levels released by male *L. callipterus*. Free 11-KT (A), free T (B), conjugated 11-KT (C) and conjugated T (D) released in pg g^{-1} h^{-1} by individuals of the different male tactics: nest males (N=10), sneaker males (N=10) and dwarf males (N=7), after spending 1 h in the mixed tactic test situation (cf. Fig. 1, exposure phase 5). Medians and interquartile ranges are shown. *P<0.05, **P<0.01, ***P<0.005 (pairwise Wilcoxon rank-sum tests; Holm-corrected P-values are provided in Table S1A,B).

densities). We corrected both fractions released per hour for sampling volume (ml) and for male body mass (g), to standardize for individuals of the three different male tactics. Correcting for body mass in males of all three male tactics resulted in an increase in the variance in the group with the smallest body mass (dwarf males) relative to the variance in the larger nest and sneaker males, because the higher correction factor for size in dwarf males amplifies the potential measurement error of the EIA kit. Nevertheless, steroid values for dwarf males are probably accurate since the EIA values appeared in the middle of the standard curve; no value emerged at its margins, where the measurement error of the EIA kit is largest. Samples sizes ranged from N=7–13, depending on the hormone fraction and treatment, and male type and treatment (see Results).

Data analysis
All statistical analyses were performed using R 3.0.2. Non-parametric Kruskal–Wallis-ANOVA and Wilcoxon rank-sum tests (R-packages 'stats') were performed to compare androgen levels of the free and the conjugated fractions among the different male tactics. Our data showed a significant correlation between body weight and 11-KT levels in both nest males and dwarf males. We therefore considered it appropriate to standardize androgen levels of all individuals by male body mass. Whereas free 11-KT and T of the nest males and intruder males of the nest male treatment (Fig. 2B) were normally distributed and therefore compared using paired *t*-tests, the conjugated fractions of these two male types were compared with Wilcoxon rank sum tests because data were non-

normally distributed. To test whether the different competitive treatments with varying male intruders (Fig. 2, exposure phases 2–5) affected androgen responsiveness in focal nest males, we modelled the free and conjugated 11- KT and T levels of different treatments with linear mixed effects models (LMEs) using the R package 'lme4' (Bates, 2005), including treatment as a fixed effect and fish identity as random effect in all models. The model was fitted with restricted maximum likelihood (REML).

In order to test whether behaviours reflect hormone levels in focal nest males, we pooled the data of all focal nest males in the competitive male treatments (exposure phases 2–5, Fig. 2) to test for a possible correlation of aggressive behaviour and androgen levels, using Spearman's rank correlation. This correlation only includes the four intruder treatments (exposure phases 2–5, Fig. 2) where nest male aggression was addressed towards other intruder males. We did not include the control treatment in this analysis, since the aggression performed by nest males in this treatment was addressed solely towards females and represents a different context (courtship behaviour) than in the other four intruder treatments (between-male aggression). Consequently, the sample sizes for the aggressive behaviours of nest males are lower than those of courtship and nest maintenance behaviours, since the control treatment was not included in this analysis. To test whether courtship and nest maintenance behaviours reflect androgen levels, we pooled the data from the competitive and non-competitive test situations (exposure phases 1–5, Fig. 2) and tested for possible correlations using Spearman's rank correlation analysis (Fox and Weisberg, 2011).
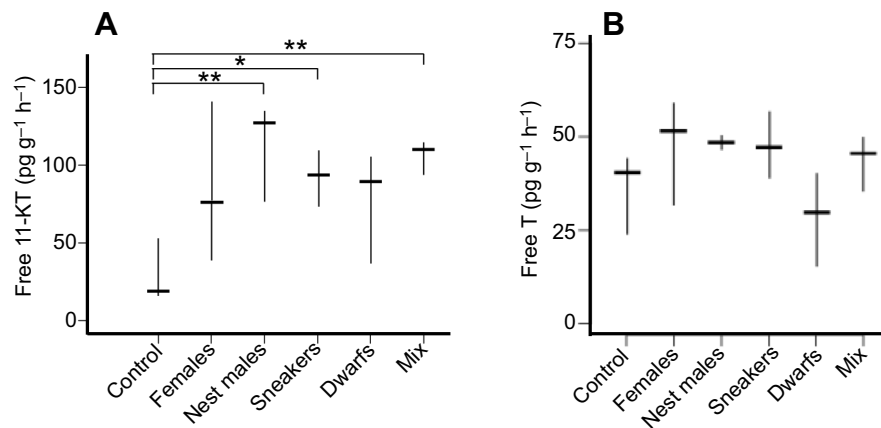
Fig. 4. Free androgen levels released by focal nest male *L. callipterus* after different competitive test situations. Free 11-KT (A) and free T (B) released by males in pg g^{-1} h^{-1} in the control situation after 1 week in isolation (11-KT, N=7; T, N=13) and in the different non-competitive and competitive test situations: with no intruder (females only: 11-KT, N=9; T, N=8), with one potential nest male intruder (11-KT, N=10; T, N=10), with two sneaker males (11-KT, N=13; T, N=12), with two dwarf males (11-KT, N=13; T, N=12) and with a combination of one sneaker and one dwarf male (11-KT, N=10; T, N=11). Medians and interquartile ranges are shown. *P<0.05, **P<0.01 (linear mixed models and multiple comparisons; Tukey-adjusted P-values are provided in Table S2A,B).

RESULTS

Androgen levels of males pursuing different ARTs

Free androgen fraction

The free fraction of 11-KT differed significantly among male tactics in the mixed tactics test situation (Fig. 2, exposure phase 5) with individuals of all three male tactics simultaneously present (Kruskal–Wallis ANOVA, H_2=10.83, P=0.0044; Fig. 3A). The dominant bourgeois males (N=10) had higher levels of free 11-KT than males pursuing the conditional sneaker male tactic (N=10) (Wilcoxon rank-sum test, W=81; Holm-adjusted P<0.05; Fig. 3A). Interestingly, parasitic dwarf males (N=7) showed the highest free 11-KT levels, significantly exceeding those of sneaker males (Wilcoxon rank-sum test, W_7=70; Holm-adjusted P<0.01; Fig. 3A), whereas these levels were not significantly different from the free 11-KT levels of nest males (Wilcoxon rank-sum test, W=22; Holm-adjusted P=0.37, Table S1A). Individuals of the three male tactics differed also in free T levels (Kruskal–Wallis ANOVA, H_2=12.0295, P=0.0024; Fig. 3B), with dwarf males (N=7) having significantly higher free T levels than nest males (N=10) (Wilcoxon rank-sum test, W=0; Holm-adjusted P<0.001; Fig. 3B) and sneaker males (N=10) (Wilcoxon rank-sum test, W=0; Holm-adjusted P<0.01; Fig. 3B); nest males [median (MD) and interquartile range (IQR): 43.78; 32.64–48.91 pg g^{-1} h^{-1}] and sneaker males (MD and IQR: 25.68; 21.598–32.45 pg g^{-1} h^{-1}) did not differ in their free T levels (Table S1A).

Conjugated androgen fraction

The conjugated fraction of 11-KT differed significantly between male tactics (Kruskal–Wallis ANOVA, H_2=16.536; P<0.001, Fig. 3C). Dwarf males (N=7) showed the highest conjugated 11-KT levels, significantly exceeding nest males (N=10) (Wilcoxon rank-sum test, W=0; P<0.001; Fig. 3C) and sneaker males (N=10) (Wilcoxon rank-sum test, W=70; P<0.001; Fig. 3C); the latter two did not differ from each other (Table S1B). The pattern of the considerably lower conjugated T levels resembled the pattern found in the free T fraction (Kruskal–Wallis ANOVA, H_2=13.907; P<0.001), with dwarf males (N=7) excreting the highest levels of conjugated T (Fig. 3D, Table S1B). Sneaker males (N=10) showed significantly higher conjugated T values than nest males (N=10) (Wilcoxon rank-sum test, W=10; P<0.01; Fig. 3D).

Androgen responses to different social challenges

Nest males

Overall, the different social challenges significantly affected the free 11-KT responses of nest males (LME: free 11-KT versus treatment, t=12.53, d.f.=5; P<0.001; Fig. 4A). Free 11-KT levels tended to increase from the control situation (after 5 days in isolation) to the

females-only exposure (breeding baseline) and from the female-only exposure to the nest male intruder challenge. While the latter response differed significantly from the control levels, they did differ from the females-only test situation (Fig. 4A; Table S2A). The free 11-KT responses of nest males did not differ from each other when exposed to different parasitic male intruders (Fig. 4A). Neither the free T levels (Fig. 4B, Table S2B), nor the conjugated fractions of 11-KT and T (Table S4A,B) differed significantly between the test situations.

Association of hormones and behaviour

There was a non-significant positive correlation between aggressive behaviour and free 11-KT levels in the competitive test situations (pooled competitive treatments: Spearman's rho=0.341, N=31; P=0.062; see Table 1, Fig. S1). No relationships between 11-KT levels and courtship or nest maintenance behaviours were detected (all treatments pooled, see Table 1). Free T levels of nest males correlated positively with aggressive behaviour (pooled treatments, Spearman's rho=0.37, N=33; P=0.019, see Table 1, Fig. 5), but not with courtship and nest maintenance behaviours.

Parasitic and nest male intruders

We tested for differential androgen responses of parasitic males between different social contexts by comparing the values of sneaker males between the sneaker male treatment with the mixed tactic treatment (Fig. 2, exposures 3 and 5). Sneaker males did not show different free 11-KT or free T responses (Table S3). They also did not behave differently in the two test situations (data not shown). Likewise, the free 11-KT and free T responses did not differ in dwarf males between the dwarf male and mixed tactic treatments (Table S3). This was also in accordance with the lack of any differences in their behaviour (data not shown). The nest male

Table 1. Relationship between hormones and behaviour in nest males of *L. callipterus*

Hormone	Behaviour	N	S	rho	P
11-KT	Aggression	31	3270	0.341	0.062
11-KT	Courting	41	11,386	0.008	0.959
11-KT	Maintenance	41	13,670.24	−0.191	0.232
T	Aggression	33	3794	0.366	**0.019**
T	Courting	40	9952	0.066	0.342
T	Maintenance	40	8485.63	0.204	0.206

Spearman's rank correlation analyses for free androgens released in pg g^{-1} h^{-1} and different nest male behavioural categories. Data are pooled from all territorial nest males and all different competitive test situations. Significant values are marked in bold, non-significant trends are underlined.
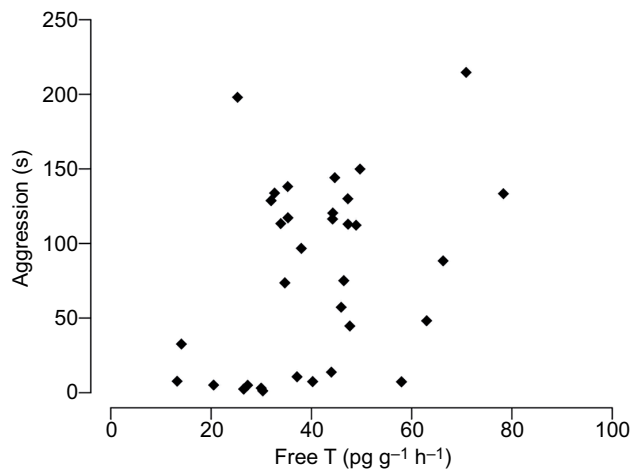
Fig. 5. Relationship of free T released and aggressive behaviour of nest males in all different competitive test situations. Data were pooled for Spearman's rank correlation analysis (Spearman's rho=0.37, N=33, P=0.019) and are shown as time displaying aggressive behaviour (s) as a function of free T level in pg g^{-1} h^{-1}.

intruders showed significantly lower free 11-KT levels than the dominant territory holders (two sample t-test: t=2.14, d.f.=17; P=0.046; Fig. S2A). Free T levels, however, did not differ between territory owners and intruders (two sample t-test: t=0.85, d.f.=17, P=0.41; Fig. S2B), and neither did the conjugated 11-KT levels (Fig. S2C). However, intruder males tended to have higher conjugated T levels (MD and IQR: 4.83; 2.78–5.26 pg g^{-1} h^{-1}) than nest owners (MD and IQR: 5.58; 4.85–13.79 pg g^{-1} h^{-1}; Wilcoxon rank-sum test: W=21; P=0.53; Fig. S2D).

DISCUSSION

In accordance with predictions of the relative plasticity hypothesis (Moore, 1991), free 11-KT levels of males with genetically fixed tactics (dwarf males and bourgeois nest males) did not differ from each other, whereas those of individuals pursuing the plastic sneaker tactic differed strongly. To our knowledge, these are the first data confirming this essential prediction of the relative plasticity hypothesis in a teleost species with both fixed and plastic alternative reproductive tactics. In previous studies, the primary androgen 11-KT (e.g. measured from plasma samples) was always higher in bourgeois males compared with individuals of the respective fixed alternative tactic (Oliveira et al., 2008). Territorial males in the corkwing wrasse (*Symphodus melops*), for instance, had significantly higher plasma levels of 11-KT than males pursuing the fixed alternative female mimic, which seemed to relate to their differences in primary and secondary sex characters (Uglem et al., 2002). A similar trend was found in the plainfin midshipman (*Porichthys notatus*; Brantley et al., 1993) and bluegill sunfish (*Lepomis macrochirus*; Kindler et al., 1989; Knapp and Neff, 2007). The similarly high levels of free 11-KT in parasitic dwarf males when compared with the bourgeois nest males in *L. callipterus* may be associated with the regulation of spermatogenesis, because dwarf males in this species apparently invest heavily in testes and sperm production (Schütz et al., 2010), which is also reflected by their extraordinary fertilization success when in direct sperm competition with bourgeois nest males (Wirtz Ocana et al., 2014). The rate of hormone release into water samples by fish has been shown to be proportional to their concentration in the plasma in several species (Scott et al., 2008), allowing us to compare these different studies with our results.

In contrast to individuals of fixed alternative reproductive tactics, the relative plasticity hypothesis predicts that adult males pursuing plastic alternative tactics should differ in their androgen levels. An activational effect of hormones during adulthood is thought to cause a switch from one tactic to another at certain stages in an individual's life (Moore, 1991). Bourgeois nest males in our study indeed had significantly higher levels of free 11-KT than plastic sneaker males, which may be associated with the clear differences in social dominance and reproductive activities between these male types, as shown in a previous study (von Kuerthy and Taborsky, 2016). This pattern of elevated free 11-KT levels in bourgeois nest males compared with opportunistic sneaker males is consistent with results from previous studies of teleosts with ARTs, such as the bluegill sunfish (Kindler et al., 1989; Knapp and Neff, 2007), peacock blenny (Oliveira et al., 2001b), Azorean rock-pool blenny (Oliveira et al., 2001c), and saddleback wrasse (Hourigan et al., 1991). We would expect that previous sneaker males in *L. callipterus* that have switched from sneaker to nest male status exhibit similar free 11-KT levels as the nest males studied here, because the sneaker male tactic is a transient stage leading to the final bourgeois phase provided that individuals survive to this stage. This has also been shown in other species with alternative reproductive tactics (e.g. Gonçalves et al., 2007). A comparison of hormone levels of *L. callipterus* sneaker males prior to and after their switch to nest male status would be an obvious target for future research. Additionally, experiments on the effects of sex steroid administration to promote differences in behaviour and physiology (e.g. a tactic switch) would strongly contribute to our understanding of hormonal regulation of male tactics in this species.

The genetically distinct dwarf males had significantly higher levels of free T than nest males and sneaker males. This is in consensus with the few previous studies on teleosts with fixed ARTs, where plasma T levels of individuals of the parasitic tactic were either higher than those of the bourgeois males (Mayer et al., 1990; Uglem et al., 2002) or they did not differ (Kindler et al., 1989; Modesto and Canário, 2003; Oliveira et al., 2003). Similar to dwarf males in *L. callipterus*, T levels in the Plainfin midshipman, for instance, were higher in males of the alternative male morph that does not court females but instead parasitizes bourgeois males (Brantley et al., 1993). Dwarf males in *L. callipterus* have been shown to not invest in courtship or aggressive behaviour, but rather try to stay inconspicuous when in the proximity of a nest male's territory (Schütz et al., 2010; von Kuerthy and Taborsky, 2016). In male striped mice (*Rhabdomys pumilio*), a species with phenotypically plastic ARTs, dominant territorial breeders also had lower T levels than the plastic, solitary roamers that are less competitive and pursue a parasitic sneaker tactic (Schradin et al., 2009). It has been suggested that the high levels of T in solitary roamers would promote risky behaviour, such as invading territories defended by dominant males. Similarly, although representing individuals of a fixed tactic and although androgen regulation differs between fish and mammals, dwarf males in *L. callipterus* are also solitary roamers, which might make use of the potentially anxiolytic, fear-reducing effects associated with elevated testosterone levels (Vandenheede and Bouissou, 1993; Aikey et al., 2002; Hermans et al., 2006; Vermeersch et al., 2008) when approaching a nest site where a spawning is taking place. The reproductive tactic of dwarf males involves risk-prone behaviours, as they invade territories of bourgeois males that are more than 40 times bigger (37.5 g versus 0.9 g; Sato et al., 2004) in order to steal fertilizations. Nest owners have been observed to kill dwarf males if they catch them (our observations).

Contrary to predictions of the relative plasticity hypothesis, free T levels did not differ between nest holders and sneaker males. This was also reported for bourgeois and parasitic males in the saddleback wrasse and rock-pool blenny (Hourigan et al., 1991; Oliveira et al., 2001c). In contrast, in the peacock blenny, both 11-KT and T levels were higher in floaters and nest holders than in sneaker males (Ros and Oliveira, 2009). This highlights the possibility that T responses in species with ARTs may be rather inconsistent (Mayer et al., 1990; Brantley et al., 1993; Cheek et al., 2000), as they may depend on the peculiar behavioural differences involved in the alternative reproductive tactics expressed in a species. Our data show a significant correlation between body mass and 11-KT levels in both nest males and dwarf males. We therefore considered it appropriate to standardize androgen levels of individuals of the different tactics by male body mass. We should like to stress, however, that it is currently unclear whether standard allometric scaling of hormone levels as measured in fish holding water is appropriate when comparing differently sized conspecifics and adequate allometric functions to correct for respective size differences are yet unknown (Bender et al., 2006). Irrespective of this open question it is, however, obvious that *L. callipterus* dwarf males produce relatively high amounts of the primary androgens.

Regarding the second part of our experiment where the social reciprocity models were tested, we predicted an increase in androgens when nest males were challenged by an intruder and different responses when they were challenged by different types of competitors. In accordance with this prediction, we found an overall increase in free 11-KT levels with rising degrees of social challenge, from the control treatment (isolation), to the females-only treatment (with shells, but without male competition) and finally to the competitive test situations (intruder males). The effect sizes were rather small (see Table S2A), which is consistent with results from previous studies (Landys et al., 2010). Furthermore, free 11-KT responses of nest males in the different competitive test situations (except in the dwarf male treatment) were significantly higher when compared with the control situation (i.e. 5 days in isolation).

In contrast to free 11-KT, free T levels of nest males did not significantly vary with the different social challenges, which is surprising given that androgens are involved in the expression of aggressive and reproductive behaviours in a large number of teleost species (Liley and Stacey, 1983; Borg, 1994). However, when all competitive test situations were combined, nest male aggression correlated positively with free T levels, whereas there was only a non-significant trend in the relationship with free 11-KT. A similar pattern was found in the cooperatively breeding cichlid *Neolamprologus pulcher*, where male aggression correlated positively with T but not with 11-KT or E2 (Desjardins et al., 2006). In our experiment, no relationships emerged of free T and 11-KT with courtship and nest maintenance behaviours.

Evolutionary theory predicts that when alternative behaviours exist in a population, competition among individuals pursuing the same tactic should be greater than between individuals pursuing different tactics (Maynard Smith, 1974; Gross, 1991). However, we observed hardly any aggressive interactions within or between the sneaker male and dwarf male tactics (see also von Kuerthy and Taborsky, 2016). Correspondingly, parasitic males did not show different androgen responsiveness when challenged with individuals of either the same or another parasitic tactic. In contrast to bourgeois males, parasitic males always face reproductive competition when spawning, as they always compete for fertilizations with nest males. Therefore, they may not need to adjust their behaviour and androgen levels to the additional presence of same or different parasitic males.

A relationship between social status and androgen levels has been demonstrated in several species for both T and 11-KT levels (Oliveira et al., 1996, 2002; Mazur and Booth, 1998; Parikh et al., 2006). In our study, the androgen levels of nest owners, who always won the contests with intruders of the bourgeois male type, also differed from those of the latter, with nest owners showing significantly higher free 11-KT levels than intruders. However, the free T levels did not differ between these males.

Interestingly, conjugated T levels tended to be slightly higher (but non-significant) in intruders of the bourgeois male type than in nest owners. Subordinate sneakers also exhibited higher levels of the conjugated T fraction than nest owners, which may indicate that sneaker males urinated more during the sampling phase than the nest owners did. Dwarf males released significantly higher levels of the conjugated 11-KT fraction than the other male tactics, bourgeois and sneaker males. Dwarf males also had significantly higher conjugated T levels than sneaker males and nest males, with the latter showing the lowest levels of all male types. Potential differences in excretion of urine and its composition may be explained by two alternative processes. (1) Urine excretion can function as an inactivation pathway. For example, the subordinate sneaker and dwarf males might eliminate T actively via excretion (conjugation) in order to diminish the production of 11-KT; T would then no longer be available as precursor of the synthesis of 11-KT. (2) Alternatively, excretion could serve to produce social cues (pheromone production; Bergman et al., 2005; Barata et al., 2007; Hirschenhauser et al., 2008; Scott et al., 2008; Martinovic-Weigelt et al., 2012). It would be counterintuitive, however, for parasitic males and intruders of the bourgeois male type to produce more of these cues than the territorial nest owners. Still another explanation might be that experimental subjects had retained urine during the experimental exposure and released the built-up urine in the subsequent measurement period.

The proximate mechanisms underlying the expression of ARTs are still poorly understood. Species exhibiting both fixed and flexible ARTs, like *L. callipterus*, are ideal to investigate the role of androgens in reproducing individuals, within-sex variation in reproductive phenotypes and the degree to which androgen responses of divergent tactics are triggered by different social challenges. In this study, important predictions of the relative plasticity hypothesis have been confirmed, although there are also clear deviations. In addition, our data reveal that androgen levels of males are influenced by specific social and reproductive challenges, confirming predictions of the social reciprocity models like the biosocial feedback model and the challenge hypothesis. Furthermore, the androgen levels of bourgeois males correlated significantly with the quantity of aggressive behaviour they showed, revealing hormonal regulation of aggression in nest owners. These results provide new insight into the endocrine responsiveness of males pursuing fixed and plastic ARTs.

Competing interests
The authors declare no competing or financial interests.

Author contributions
C.v.K.: Experimental design, data acquisition, laboratory and statistical analyses, writing the manuscript. A.F.H.R.: Provisioning of laboratory facilities, supervision of laboratory work, help with planning the study and writing the manuscript. M.T.: Design of study and writing the manuscript, supervision during all stages of the study.

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